

PRELIMINARY AMENDMENT

Serial Number: 09/637,614

Filing Date: August 11, 2000

Title: ARTIFICIAL ANTIBODY POLYPEPTIDES

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Remarks

Claims 15-29 and 36-37 are pending.

The Commissioner is hereby authorized to charge any additional fees to Deposit Account No: 19-0743.

When this application is taken up for the first Office Action, Applicant respectfully requests that the Examiner consider this Preliminary Amendment.

Respectfully submitted,

SHOHEI KOIDE

By their Representatives,

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Date 23 July 2001

By

Ann S. Viksnins

Reg. No. 37,748

CERTIFICATE UNDER 37 CFR 1.8: The undersigned hereby certifies that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail, in an envelope addressed to: Commissioner of Patents, Washington, D.C. 20231, on this 23 day of July, 2001.

Candis B. Buending

Name

Signature

Candis B. Buending

CLEAN VERSIONS OF SUBSTITUTE PARAGRAPHS IN THE SPECIFICATION

Please substitute page 12, second paragraph for the following paragraphs:

B
Figure 1A. β -Strand and loop topology of anti-lysozyme immunoglobulin D1.3. (Bhat et al., 1994) The locations of complementarity determining regions (CDRs, hypervariable regions) are indicated.

Figure 1B. β -Strand and loop topology of the 10th type III domain of human fibronectin. (Main et al., 1992) The locations of the integrin-binding Arg-Gly-Asp (RGD) sequence is indicated.

Figure 1C. MOLSCRIPT representation of anti-lysozyme immunoglobulin D1.3. (Kraulis, 1991; Bhat et al., 1994) The locations of complementarity determining regions (CDRs, hypervariable regions) are indicated.

Figure 1D. MOLSCRIPT representation of the 10th type III domain of human fibronectin. (Kraulis, 1991; Main et al., 1992) The locations of the integrin-binding Arg-Gly-Asp (RGD) sequence is indicated.

Please substitute page 12, fourth paragraph for the following paragraphs:

B²
Figure 3A. Far UV CD spectra of wild-type Fn3 at 25°C and 90°C. Fn3 (50 μ M) was dissolved in sodium acetate (50 mM, pH 4.6).

Figure 3B. Thermal denaturation of Fn3 monitored at 215 nm. Temperature was increased at a rate of 1°C/min.

Please substitute the fifth paragraph on page 12 continuing on page 13 for the following paragraphs:

B³
Figure 4A. C_{α} trace of the crystal structure of the complex of lysozyme (HEL) and the Fv fragment of the anti-hen egg-white lysozyme (anti-HEL) antibody D1.3 (Bhat et al., 1994). Side chains of the residues 99-102 of VH CDR3, which make contact with HEL, are also shown.

Figure 4B. Contact surface area for each residue of the D1.3 VH-HEL and VH-VL interactions plotted vs. residue number of D1.3 VH. Surface area and secondary structure were determined using the program DSSP (Kabsh and Sander, 1983).

Figure 4C. Schematic drawings of the β -sheet structure of the F strand-loop-G strand moieties of D1.3 VH. The boxes denote residues in β -strands and ovals those not in strands. The shaded boxes indicate residues of which side chains are significantly buried. The broken lines indicate hydrogen bonds.

Figure 4D. Schematic drawings of the β -sheet structure of the F strand-loop-G strand moieties of Fn3. The boxes denote residues in β -strands and ovals those not in strands. The shaded boxes indicate residues of which side chains are significantly buried. The broken lines indicate hydrogen bonds.

Please substitute page 13, paragraph 5 for the following paragraph:

B4
Figure 9. (Ubiquitin-1) Characterization of ligand-specific binding of enriched clones using phage enzyme-linked immunosorbent assay (ELISA). Microtiter plate wells were coated with ubiquitin (1 µg/well; "Ligand (+)) and then blocked with BSA. Phage solution in TBS containing approximately 10^{10} colony forming units (cfu) was added to a well and washed with TBS. Bound phages were detected with anti-phage antibody-POD conjugate (Pharmacia) with Turbo-TMB (Pierce) as a substrate. Absorbance was measured using a Molecular Devices SPECTRAMAX 250 microplate spectrophotometer. For a control, wells without the immobilized ligand were used. 2-1 and 2-2 denote enriched clones from Library 2 eluted with free ligand and acid, respectively. 4-1 and 4-2 denote enriched clones from Library 4 eluted with free ligand and acid, respectively.

Please substitute page 14, fifth paragraph for the following paragraphs:

B5
Figure 15A. Characterization of the binding reaction of Ubi4-Fn3 to the target, ubiquitin. Phage ELISA analysis of binding of Ubi4-Fn3 to ubiquitin. The binding of Ubi4-phages to ubiquitin-coated wells was measured. The control experiment was performed with wells containing no ubiquitin.

Figure 15B. Competition phage ELISA of Ubi4-Fn3. Ubi4-Fn3-phages were preincubated with soluble ubiquitin at an indicated concentration, followed by the phage ELISA detection in ubiquitin-coated wells.

Figure 15C. Competition phage ELISA testing the specificity of the Ubi4 clone. The Ubi4 phages were preincubated with 250 µg/ml of soluble proteins, followed by phage ELISA as in (b).

Figure 15D. ELISA using free proteins.

Please substitute page 23, paragraph 1 for the following paragraph:

B4
Fn3 without His•tag was purified as follows. Cells were suspended in 5 ml/(g cell) of Tris (50 mM, pH 7.6) containing ethylenediaminetetraacetic acid (EDTA; 1 mM) and phenylmethylsulfonyl fluoride (1 mM). HEL was added to a final concentration of 0.5 mg/ml. After incubating the solution for 30 minutes at 37°C, it was sonicated three times for 30 seconds on ice. Cell debris was removed by centrifugation. Ammonium sulfate was added to the solution and precipitate recovered by centrifugation. The pellet was dissolved in 5-10 ml sodium acetate (50 mM, pH 4.6) and insoluble material was removed by centrifugation. The solution was applied to a SEPHACRYL S100HR column (Pharmacia) equilibrated in the sodium acetate buffer. Fractions containing Fn3 then was applied to a RESOURCES® column (Pharmacia) equilibrated in sodium acetate (50 mM, pH 4.6) and eluted with a linear gradient of sodium chloride (0-0.5 M). The protocol can be adjusted to purify mutant proteins with different surface charge properties.

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Please substitute page 23, paragraph 2 for the following paragraph:

Fn3 with His•tag was purified as follows. The soluble fraction was prepared as described above, except that sodium phosphate buffer (50 mM, pH 7.6) containing sodium chloride (100 mM) replaced the Tris buffer. The solution was applied to a HI-TRAP chelating column (Pharmacia) preloaded with nickel and equilibrated in the phosphate buffer. After washing the column with the buffer, His•tag-Fn3 was eluted in the phosphate buffer containing 50 mM EDTA. Fractions containing His•tag-Fn3 were pooled and applied to a SEPHACRYL S100-HR column, yielding highly pure protein. The His•tag portion was cleaved off by treating the fusion protein with thrombin using the protocol supplied by Novagen. Fn3 was separated from the His•tag peptide and thrombin by a RESOURCES® column using the protocol above.

Please substitute the third paragraph on page 23 continuing on page 24 for the following paragraph:

The wild-type and two mutant proteins so far examined are expressed as soluble proteins. In the case that a mutant is expressed as inclusion bodies (insoluble aggregate), it is first examined if it can be expressed as a soluble protein at lower temperature (e.g., 25-30°C). If this is not possible, the inclusion bodies are collected by low-speed centrifugation following cell lysis as described above. The pellet is washed with buffer, sonicated and centrifuged. The inclusion bodies are solubilized in phosphate buffer (50 mM, pH 7.6) containing guanidinium chloride (GdnCl, 6 M) and will be loaded on a HI-TRAP chelating column. The protein is eluted with the buffer containing GdnCl and 50 mM EDTA.

Please substitute page 25, first full paragraph for the following paragraph:

B?

Structures of Abs were analyzed using quantitative methods (e.g., DSSP (Kabsch & Sander, 1983) and PDBFIT (D. McRee, The Scripps Research Institute)) as well as computer graphics (e.g., QUANTA (Molecular Simulations) and WHATIF (G. Vriend, European Molecular Biology Laboratory)) to superimpose the strand-loop-strand structures of Abs and Fn3.

Please substitute page 26, paragraph 1 for the following paragraph:

The stability of FnAbs was determined by measuring temperature- and chemical denaturant-induced unfolding reactions (Pace et al., 1989). The temperature-induced unfolding reaction was measured using a circular dichroism (CD) polarimeter. Ellipticity at 222 and 215 nm was recorded as the sample temperature was slowly raised. Sample concentrations between 10 and 50 µM were used. After the unfolding baseline was

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established, the temperature was lowered to examine the reversibility of the unfolding reaction. Free energy of unfolding was determined by fitting data to the equation for the two-state transition (Becktel & Schellman, 1987; Pace et al., 1989). Nonlinear least-squares fitting was performed using the program IGOR (WaveMetrics) on a Macintosh computer.

Please substitute page 27, first full paragraph for the following paragraph:

B6

Once the reversibility of the thermal unfolding reaction is established, the unfolding reaction is measured by a Microcal MC-2 differential scanning calorimeter (DSC). The cell (~ 1.3 ml) will be filled with FnAb solution (0.1 - 1 mM) and ΔC_p ($= \Delta H / \Delta T$) will be recorded as the temperature is slowly raised. T_m (the midpoint of unfolding), ΔH of unfolding and ΔG of unfolding is determined by fitting the transition curve (Privalov & Potekhin, 1986) with the ORIGIN software provided by Microcal.

Please substitute the fourth paragraph on page 27 continuing on page 28 for the following paragraph:

B7

The enthalpy change (ΔH) of binding were measured using a Microcal OMEGA ITC (Wiseman et al., 1989). The sample cell (~ 1.3 ml) was filled with FnAbs solution ($\leq 100 \mu\text{M}$, changed according to K_d), and the reference cell filled with distilled water; the system was equilibrated at a given temperature until a stable baseline is obtained; 5-20 μl of ligand solution ($\leq 2 \text{ mM}$) was injected by a motor-driven syringe within a short duration (20 sec) followed by an equilibration delay (4 minutes); the injection was repeated and heat generation/absorption for each injection was measured. From the change in the observed heat change as a function of ligand concentration, ΔH and K_d was determined (Wiseman et al., 1989). ΔG and ΔS of the binding reaction was deduced from the two directly measured parameters. Deviation from the theoretical curve was examined to assess nonspecific (multiple-site) binding. Experiments were also performed by placing a ligand in the cell and titrating with an FnAb. It should be emphasized that only ITC gives direct measurement of ΔH , thereby making it possible to evaluate enthalpic and entropic contributions to the binding energy. ITC was successfully used to monitor the binding reaction of the D1.3 Ab (Tello et al., 1993; Bhat et al., 1994).

Please substitute the second paragraph on page 32 continuing on page 33 for the following paragraph:

B10

The binding affinity of FnAbs on phage surface is characterized semi-quantitatively using the phage ELISA technique (Li et al., 1995). Wells of microtiter plates (Nunc) are coated with a ligand protein (or with streptavidin followed by the binding of a biotinylated ligand) and blocked with the BLOTTO solution (Pierce). Purified phages ($\sim 10^{10}$ pfu) originating from single plaques (M13)/colonies (fUSE5) are

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added to each well and incubated overnight at 4°C. After washing wells with an appropriate buffer (see above), bound phages are detected by the standard ELISA protocol using anti-M13 Ab (rabbit, Sigma) and anti-rabbit Ig-peroxidase conjugate (Pierce) or using anti-M13 Ab-peroxidase conjugate (Pharmacia). Colormetric assays are performed using TMB (3,3',5,5'-tetramethylbenzidine, Pierce). The high affinity of protein G to immunoglobulins present a special problem; Abs cannot be used in detection. Therefore, to detect anti-protein G FnAbs, fusion phages are immobilized in wells and the binding is then measured using biotinylated protein G followed by the detection using streptavidin-peroxidase conjugate.

Please substitute the second paragraph on page 34 continuing on page 35 for the following paragraph:

B⁴

The gene was assembled in the following manner. First, the gene sequence (Fig. 5) was divided into five parts with boundaries at designed restriction sites: fragment 1, NdeI-PstI (oligonucleotides FN1F and FN1R (Table 2); fragment 2, PstI-EcoRI (FN2F and FN2R); fragment 3, EcoRI-SalI (FN3F and FN3R); fragment 4, SalI-SacI (FN4F and FN4R); fragment 5, SacI-BamHI (FN5F and FN5R). Second, for each part, a pair of oligonucleotides which code opposite strands and have complementary overlaps of approximately 15 bases was synthesized. These oligonucleotides were designated FN1F-FN5R and are shown in Table 2. Third, each pair (e.g., FN1F and FN1R) was annealed and single-strand regions were filled in using the Klenow fragment of DNA polymerase. Fourth, the double stranded oligonucleotide was digested with the relevant restriction enzymes at the termini of the fragment and cloned into the PBLUESCRIPT SK plasmid (Stratagene) which had been digested with the same enzymes as those used for the fragments. The DNA sequence of the inserted fragment was confirmed by DNA sequencing using an Applied Biosystems DNA sequencer and the dideoxy termination protocol provided by the manufacturer. Last, steps 2-4 were repeated to obtain the entire gene.

Please substitute page 37, paragraph 2 for the following paragraph:

B¹²

Site-directed mutagenesis is performed following published methods (see for example, Kunkel, 1985) using a MUTA-GENE kit (BioRad). The libraries are constructed by electroporation of *E. coli* XL-1 Blue electroporation competent cells (200 µl; Stratagene) with 1 µg of the plasmid DNA using a BTX electrocell manipulator ECM 395 1mm gap cuvette. A portion of the transformed cells is plated on an LB-agar plate containing ampicillin (100 µg/ml) to determine the transformation efficiency. Typically, 3×10^8 transformants are obtained with 1 µg of DNA, and thus a library contains 10^8 to 10^9 independent clones. Phagemid particles were prepared as described above.

Please substitute the second paragraph on page 41 continuing on page 42 for the following paragraph:

B13
Fn3 and monobodies with His•tag were purified as follows. Cells were suspended in 5 ml/(g cell) of 50 mM Tris (pH 7.6) containing 1 mM phenylmethylsulfonyl fluoride. HEL (Sigma, 3X crystallized) was added to a final concentration of 0.5 mg/ml. After incubating the solution for 30 min at 37°C, it was sonicated so as to cause cell breakage three times for 30 seconds on ice. Cell debris was removed by centrifugation at 15,000 rpm in an Sorval RC-2B centrifuge using an SS-34 rotor. Concentrated sodium chloride is added to the solution to a final concentration of 0.5 M. The solution was then applied to a 1 ml HISTRAP™ chelating column (Pharmacia) preloaded with nickel chloride (0.1 M, 1 ml) and equilibrated in the Tris buffer (50 mM, pH 8.0) containing 0.5 M sodium chloride. After washing the column with the buffer, the bound protein was eluted with a Tris buffer (50 mM, pH 8.0) containing 0.5 M imidazole. The His•tag portion was cleaved off, when required, by treating the fusion protein with thrombin using the protocol supplied by Novagen (Madison, WI). Fn3 was separated from the His•tag peptide and thrombin by a RESOURCES® column (Pharmacia) using a linear gradient of sodium chloride (0 - 0.5 M) in sodium acetate buffer (20 mM, pH 5.0).

Please substitute the third paragraph on page 43 continuing on page 44 for the following paragraph:

B14
Target molecules were immobilized in wells of a microtiter plate (MAXISORP, Nunc) as described hereinbelow, and the wells were blocked with BSA. In addition to the use of carrier protein as described below, a conjugate of a target molecule in biotin can be made. The biotinylated ligand can then be immobilized to a microtiter plate well which has been coated with streptavidin.

Please substitute page 47, paragraph 2 for the following paragraph:

B15
NMR experiments are performed on a Varian Unity INOVA 600 spectrometer equipped with four RF channels and a triple resonance probe with pulsed field gradient capability. NMR spectra are analyzed using processing programs such as FELIX (Molecular Simulations), NMRPIPE, PIPP, and CAPP (Garrett, *et al.*, 1991; Delaglio, *et al.*, 1995) on UNIX workstations. Sequence specific resonance assignments are made using well-established strategy using a set of triple resonance experiments (CBCA(CO)NH and HNCACB) (Grzesiek & Bax, 1992; Wittenkind & Mueller, 1993).

Please substitute page 49, first full paragraph for the following paragraph:

Ble
Backbone ^1H , ^{15}N and ^{13}C resonance assignments for a monobody are compared to those for wild-type Fn3 to assess structural changes in the mutant. Once these data establish that the mutant retains the global structure, structural refinement is performed using experimental NOE data. Because the structural difference of a monobody is expected to be minor, the wild-type structure can be used as the initial model after modifying the amino acid sequence. The mutations are introduced to the wild-type structure by interactive molecular modeling, and then the structure is energy-minimized using a molecular modeling program such as QUANTA (Molecular Simulations). Solution structure is refined using cycles of dynamical simulated annealing (Nilges *et al.*, 1988) in the program X-PLOR (Brünger, 1992). Typically, an ensemble of fifty structures is calculated. The validity of the refined structures is confirmed by calculating a fewer number of structures from randomly generated initial structures in X-PLOR using the YASAP protocol (Nilges, *et al.*, 1991). Structure of a monobody-ligand complex is calculated by first refining both components individually using intramolecular NOEs, and then docking the two using intermolecular NOEs.

Please substitute the fourth paragraph on page 53 continuing on page 54 for the following paragraph:

B17
Proteins were purified as follows. Cells were suspended in 5 ml/(g cell) of Tris (50 mM, pH 7.6) containing phenylmethylsulfonyl fluoride (1 mM). Hen egg lysozyme (Sigma) was added to a final concentration of 0.5 mg/ml. After incubating the solution for 30 minutes at 37°C, it was sonicated three times for 30 seconds on ice. Cell debris was removed by centrifugation. Concentrated sodium chloride was added to the solution to a final concentration of 0.5 M. The solution was applied to a HI-TRAP chelating column (Pharmacia) preloaded with nickel and equilibrated in the Tris buffer containing sodium chloride (0.5 M). After washing the column with the buffer, histag-Fn3 was eluted with the buffer containing 500 mM imidazole. The protein was further purified using a RESOURCES® column (Pharmacia) with a NaCl gradient in a sodium acetate buffer (20 mM, pH 4.6).

Please substitute page 57, first full paragraph for the following paragraph:

Ubi4-Fn3 was dissolved in -Gly HCl buffer (20 mM, pH 3.3) containing NaCl (300 mM) using an Amicon ultrafiltration unit. The final protein concentration was 1 mM. NMR experiments were performed on a Varian Unity INOVA 600 spectrometer equipped with a triple-resonance probe with pulsed field gradient. The probe temperature was set at 30°C. HSQC, TOCSY-HSQC and NOESY-HSQC spectra were recorded using published procedures (Kay *et al.*, 1992; Zhang *et al.*, 1994). NMR spectra were processed and analyzed using the NMRPIPE and NMRVIEW software (Johnson & Blevins, 1994; Delaglio *et al.*, 1995) on UNIX workstations. Sequence-specific resonance assignments

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were made using standard procedures (Wüthrich, 1986; Clore & Gronenborn, 1991). The assignments for wild-type Fn3 (Baron et al., 1992) were confirmed using a ^{15}N -labeled protein dissolved in sodium acetate buffer (50 mM, pH 4.6) at 30°C.
